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Abnormal Sea Urchin Fertilization Envelope Assembly in Low Sodium Seawater

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Abstract. The structuralization of the sea urchin fertilization envelope (FE), a model for extracellular macromolecular assembly, was found to require sodium ions, the predominant cation of seawater. Eggs from *Strongylocentrotus purpuratus* activated in sea waters with sodium chloride substitutes (choline or Tris chloride) elevated incomplete FEs. In addition, the conversion of the microvillar casts of the FE from blunt (I-form) to angular (T-form) did not occur. The permeability of the abnormal FEs was also compromised, as approximately eight times more protein than normal was released into the ambient seawater. There were also significant increases in the escape of two cortical granule (CG) enzymes, β-1,3-glucanase and ovoperoxidase. Furthermore, FEs elevated in choline chloride (ChCl) seawater appeared to be deficient in the incorporation of ovoperoxidase, an enzyme that is normally bound to the FE and that cross-links structural proteins in the nascent FE. The morphology of FEs elevated in potassium chloride-substituted seawater was similar to those in normal sodium seawater. Thus, it appears that sodium, or at least a similar ion, is necessary for the proper functioning of ovoperoxidase and structural proteins in the elevation and normal assembly of the sea urchin FE.

Introduction

Sea urchin fertilization has been intensively studied for the dramatic intra- and extracellular events concomitant with the change from egg to embryo. The irreversible transformation of the relatively thin, soft vitelline layer (VL = glycocalyx), investing the unfertilized egg, into a hardened, insoluble fertilization envelope (FE), elevated from the egg surface, is a critical step for the protection of the developing embryo. Furthermore, this process has been the subject of numerous investigations as an example of regulated extracellular matrix assembly. Several recent reviews (Kay and Shapiro, 1985; Shapiro *et al.*, 1989; Somers and Shapiro, 1989) contain the details of such investigations; therefore, the process will be summarized only briefly here.

Transglutaminase has an important role in the earliest stages of VL modification (Battaglia and Shapiro, 1988). It is located on the egg surface and catalyzes the incorporation of primary amines into the nascent FE during the first 4 min of egg activation. This process is apparently related to the I-T transition of the microvillar projections of the VL (in *S. purpuratus*), because the transition does not occur in the presence of transglutaminase inhibitors. During the next few minutes, ovoperoxidase secreted from the cortical granules (CG) catalyzes the insertion of structural proteins from the CGs into the VL by cross-linking tyrosyl residues between polypeptides (Foerder and Shapiro, 1977; Hall, 1978). Besides this catalytic reaction, ovoperoxidase itself is incorporated into the nascent FE via a specific interaction with another CG protein, proteoliaisin (Weidman *et al.*, 1985). These enzyme activities result in a hardened, insoluble, fully formed FE within the first 10 min following egg activation.

Several recent reports have been concerned with ionic requirements for FE formation. Carroll and Endress (1982) demonstrated that formation of mature FEs requires the normal 9 m*M* [Ca^{2+}] and 48 m*M* [Mg^{2+}] in the seawater. By omitting these ions they could produce an "intermediate envelope" that was much thinner than the normal FE and did not incorporate structural proteins.

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Abbreviations: SW (artificial seawater); CG (cortical granule); ChCl (choline chloride); FE (fertilization envelope); FP (fertilization product); VL (vitelline layer).

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Deficiency of Cl^-, the most abundant anion in seawater, not only interferes with the normal I-T transformation, but also increases the permeability of the FE (Lynn *et al.*, 1988; Green *et al.*, 1990). Furthermore, the normal external Na^+ concentration (419 m*M*) is not only essential for preventing polyspermy and hardening the FE, but also for the normal embryonic development of the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus* (Schuel *et al.*, 1982).

To better understand the mechanism of FE elevation—an important early event of embryonic development—we concentrated on the effect of Na^+ deficiency on the formation of the FE. In the present study, the elevation and morphology of FEs from normal and low sodium (2 m*M*) seawaters were investigated. Envelopes were observed with phase, scanning, and transmission electron microscopy. Total soluble secreted protein and the enzymatic activities of ovoperoxidase (Foerder and Shapiro, 1977; Hall, 1978) and β-1,3-glucanase (Schuel *et al.*, 1972; Wessel *et al.*, 1987) in the fertilization products (FP) were compared. Portions of this investigation have been presented in a preliminary form (Cheng *et al.*, 1989).

Materials and Methods

Handling of gametes

Sea urchin (*S. purpuratus*) eggs were collected and acid dejellied as described previously (Green *et al.*, 1990). Eggs were divided into four equal portions, the three low Na^+ groups: KCl-, Tris-, and ChCl-substituted seawaters (SW) and the control: normal Na^+-SW group. They were washed and incubated in the appropriate SW for 15–40 min. All seawater formulations were based on Cavanaugh (1956). For the low Na^+-SWs, equimolar concentrations of KCl, Tris HCl, or ChCl (Sigma) replaced NaCl, yielding a calculated residual [Na^+] of approximately 2 m*M*. Normal and low Na^+ SWs were buffered with 10 m*M* TAPS (Sigma) and adjusted to pH 8.3 with NaOH or KOH, respectively. To avoid contamination from sperm proteins and secretions, eggs were activated by adding the Ca^{2+} ionophore A23187 (Chambers *et al.*, 1974; Steinhardt and Epel, 1974) to a final concentration of 38 μ*M* in 1% dimethyl sulfoxide (DMSO). Experiments were performed at 20°C.

Light microscopy

Eggs activated in normal and low Na^+-SWs were observed continuously on a 1 × 3 inch microscope slide under a coverslip supported by a ring of petroleum jelly. Photographs were taken with phase contrast optics on Kodak Technical Pan Film 2415.

Ovoperoxidase localization

Activated eggs of the control group (normal Na^+-SW) were rinsed at *15 min postactivation* with 0.45 *M* NaCl–0.1 *M* Tris HCl (Klebanoff *et al.*, 1979). Ten percent egg suspensions (v/v) were made and incubated in 5.6 m*M* 3,3-diaminobenzidine (DAB; Sigma) in 0.45 *M* NaCl–0.1 *M* Tris HCl in an ice bath for *10 min*. The experimental eggs (low Na^+) were handled identically except that NaCl was replaced with ChCl. Activated eggs were then fixed and prepared for TEM as described below.

Transmission electron microscopy

Eggs were fixed with 2% glutaraldehyde in the appropriate seawaters for 1 h at room temperature and washed with 0.1 *M* sodium cacodylate (pH 7.4). Postfixation with 1% osmium tetroxide (OsO_4) was performed for 30 min. Eggs were washed with double distilled water and dehydrated in ascending concentrations of ethanol. Ethanol was replaced with propylene oxide and eggs were infiltrated with EM bed-812 (Electron Microscopy Sciences). The blocks were cured at 58–60°C for 3 days. Sections were cut with glass knives on a Reichert-Jung Ultracut E; mounted on copper grids; stained with lead citrate (Reynolds, 1963) and uranyl acetate; and observed with a Philips 301 transmission electron microscope at 60 kV.

Scanning electron microscopy

Fixations were accomplished at 20°C by mixing equal volumes of eggs with 4% glutaraldehyde in seawater (with 10 m*M* TAPS pH 8.3) before activation and at 1, 3, 10, 30, and 60 min postactivation. Eggs were fixed for 1 h and washed in 0.1 *M* sodium cacodylate (pH 7.4). Postfixation took place in 1% OsO_4 in 0.1 *M* sodium cacodylate (pH 7.4) for 30 min. They were then washed with double distilled water and dehydrated in an ascending series of ethanol. Absolute ethanol was replaced gradually by acetone. Eggs were transferred to porous containers (Bio-Rad) and processed for CO_2 critical point drying (Samdri-790, Tousimis Research Corp.). The eggs were then attached to aluminum mounts coated with colloidal silver liquid (Ted Pella, Inc.) for sputter coating with gold: palladium (60:40; Electron Microscopy Sciences) for 3 min in a Hummer VI (Technics). Eggs were observed with a JEOL JSM-35CF scanning electron microscope at 25 kV and a condenser lens setting of 3. The photos were taken with Polaroid type 55 (4 × 5 in.) positive/negative instant sheet film.

Total protein assay

Ionophore-activated eggs were allowed to settle for approximately 10 min and the supernatant (secreted FP) was collected and centrifuged by hand to remove the few remaining eggs. Ionophore activation resulted in at least 95% elevated FEs. For protein determination the proteins in 1.0 ml of FP (5% egg suspension, v/v) were precipitated

by adding 110 μl ice-cold 50% trichloroacetic acid (TCA) and centrifuged for 20 min at 8800 × *g* (Eppendorf centrifuge 5413) at 10°C. Tubes were drained by inversion and the protein pellets air dried. Bovine serum albumin (BSA; Sigma) was the standard. All the precipitates were assayed according to Lowry *et al.* (1951). As a control, protein determinations were performed with BSA in each of the seawaters to ascertain their influences, if any, on the Lowry procedure.

β-1,3-glucanase assay

Glucanase activity was measured according to Green and Summers (1980). The FP was collected as described above. FP (0.2 ml; 5% egg suspension, v/v), normal Na^+- or ChCl-substituted SW (0.2 ml), and laminarin (0.2 ml of a 2.5 mg/ml SW solution) were incubated for 1 h at 37°C. Then 0.2 ml of this mixture, 0.4 ml enzyme solution (0.4 ml glucose oxidase and 3 mg horseradish peroxidase in 50 ml of 25 m*M* phosphate buffer, pH 6) and 0.4 ml o-dianisidine (40 mg in 50 ml double distilled water) were incubated for 10 min at 37°C. The reaction was stopped by the addition with rapid vortexing of 0.8 ml 4 *N* sulfuric acid. Spectrophotometric readings were taken at 530 nm. Controls lacking the substrate laminarin were used to check for the presence of glucose (the product of the glucanase-laminarin reaction) in the FP. Glucose was generated *only* in the presence of both the FP and laminarin. A glucose solution was the standard. Control glucose determinations were performed in normal Na^+-SW and ChCl-substituted-SW to ascertain the effects, if any, of ChCl substitution on the glucose oxidase-peroxidase reaction. All the chemicals for this assay were purchased from Sigma.

Ovoperoxidase assay

Ovoperoxidase assays were performed in 1 ml containing 18 m*M* guaiacol (Sigma), 0.3 m*M* H_2O_2 (Sigma), and 10 m*M* TAPS at pH 8.0 and 20°C (Deits *et al.*, 1984). The reaction was started by adding enzyme (in the FP), and the increase in absorbency at 436 nm was recorded spectrophotometrically with a strip chart recorder. All reported values are initial rates, because the reaction slows after 15–30 s. A unit of ovoperoxidase was defined as that which is required to oxidize 1 μmole of guaiacol per min in a 1-ml assay volume (Deits *et al.*, 1984).

FP including the ovoperoxidase was collected from a 5% (v/v) suspension of activated eggs. Ten minutes after activation, eggs were settled by low speed centrifugation and the supernatant (FP) was removed for the assay.

Statistics

Statistical analyses for total protein, β-1,3-glucanase and ovoperoxidase assays were performed using the Student's *t* test.

Results

Observations with light microscopy

The elevation of FEs in normal and low Na^+-SWs are compared in Figure 1. Figure 1A–D depicts eggs at 1 min postactivation. Although difficult to quantify from the photomicrographs in the normal Na^+-SW control group (Fig. 1A, E), the FE appeared relatively thin at the end of the first minute and thickened with increasing time. However, it appeared thicker (more refractive) than the low Na^+ groups, especially Tris and ChCl (Fig. 1C, D). At later time points, the FE of the control group remained more refractive than those of the low Na^+ groups. These differences were more striking at 3 min postactivation when the FE of ChCl eggs began to shrink and some collapsed, while those of the control remained spherical. By 30 min postactivation, the FEs remained robust in the normal and K^+-substituted SWs (Fig. 1E, F), while those of the Tris- and choline-substituted SWs had collapsed back nearer to the egg surface (Fig. 1G, H).

An interesting attribute of the activated eggs is that of their increased stickiness in the Tris and ChCl groups. In the first 3 min there was no apparent difference among the 4 groups. At approximately 4 min postactivation, however, the eggs of the Tris and ChCl groups formed extensive clumps.

Ultrastructural changes

Unactivated eggs incubated in normal Na^+- or ChCl-substituted-SWs and observed by SEM displayed similar surface morphology (Fig. 2A, E). At 1 min postactivation, the FEs in both SWs were elevated with rounded (I-form) microvillar projections (Fig. 2B, F). In contrast to FEs in ChCl-substituted SW (Fig. 2G), the typical I-T ("Igloo-Tent") transformation of *S. purpuratus* FEs in normal Na^+-SW was completed by 3 min (Fig. 2C) and resembled those of later time points (Fig. 2D). However, ChCl FEs did not undergo the transformation even by 30 min (Fig. 2H).

As judged with TEM (Fig. 3), FEs that elevated in normal Na^+-SW resulted in the well-defined, angular T-form projections (Fig. 3A) characteristic of this species. However, those in K^+-substituted-SW appeared to be intermediate in form (Fig. 3B), compared to those in Tris- and ChCl-substituted SWs, which were similar to each other in retaining rounded projections (Fig. 3C, D).

The above observations of the "soft" FEs (incomplete formation) suggested that their permeability, as well as their morphology, might be altered. Therefore, several measurements of permeability were undertaken.

Total protein secretion

Soluble secreted protein that leaked through the FEs of eggs activated in normal or low Na^+ SWs was measured

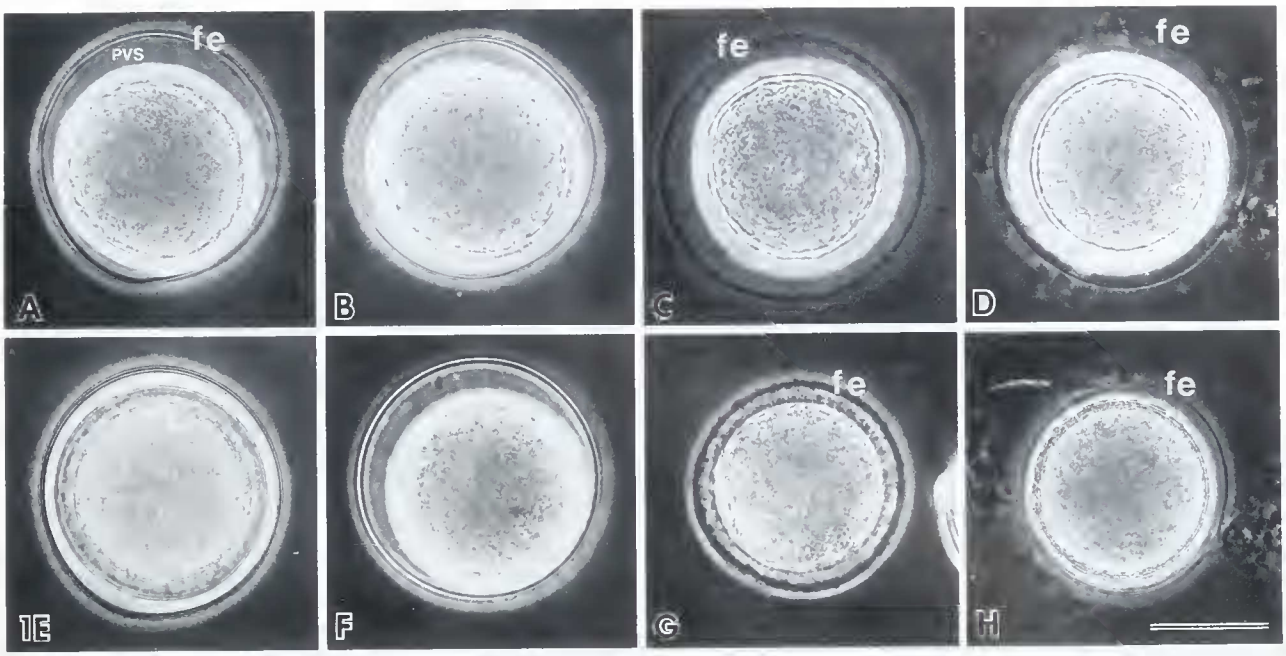

Figure 1. Phase microscopy of *Strongylocentrotus purpuratus* eggs activated in normal (A, E) and Na^+ depleted SWs (B–D and F–H). A, E: Normal Na^+-SW. B, F: KCl-substituted-SW. C, G: Tris-substituted-SW. D, H: ChCl-substituted-SW. A–D: 1 min postactivation. E–H: 30 min. These micrographs were taken focusing on the fertilization envelopes. fe = fertilization envelope; PVS = perivitelline space. Scale bar = 50 μm.

(Fig. 4). FPs were collected at 10 min postactivation from eggs pooled from several females. The FPs of the normal and K^+-substituted SW eggs had 22.7 ± 1.8 μg and 22.2 ± 5.7 μg (Mean ± S.E.M.) of protein/ml FP, respectively. They were not significantly different. However, the FPs of Tris- and ChCl-substituted SWs contained 162.1 ± 25.9 μg and 168.7 ± 3.4 μg, respectively. This 7- to 8-fold increase over normal and KCl was highly significant ($P < 0.0001$).

Control assays demonstrated no significant difference (95% confidence level) in TCA-precipitable BSA between normal and low Na^+ SWs. Therefore, the various SWs had no adverse effects on the Lowry assay. In addition, supernatant protein from *unactivated* eggs in DMSO was measured and found to contribute little to the total (~1.4 μg/ml; see also Green *et al.*, 1990).

β-1,3-glucanase secretion

Glucanase activity was measured (in normal and ChCl SWs) by the amount of glucose hydrolyzed from the β-1,3-glucan polysaccharide laminarin by egg-derived-glucanase in the FP (Fig. 5). Aliquots of FP of experimental and control groups were taken at 10 min postactivation. The glucose measurements from ChCl and normal SW were 1.62 ± 0.19 and 0.93 ± 0.19 μmoles glucose per ml of FP, respectively. Approximately 75% more glucanase activity was found in the FP from the ChCl eggs. This difference was significant ($P < 0.05$).

Control incubations of glucose were assayed in normal and ChCl-substituted SWs, and no significant differences (95% confidence level) were observed. Therefore, it is unlikely that the ChCl interfered with the glucose determination.

Ovoperoxidase secretion

Ovoperoxidase released from ChCl-SW eggs (3.72 ± 0.78 μmoles guaiacol oxidized/min/ml of FP) had significantly higher activity (see Fig. 6) than that released from normal SW eggs (0.82 ± 0.21 μmole/min/ml). Peroxidase activities in KCl-SW (1.09 ± 0.30 μmoles/min/ml) and Tris-SW (1.68 ± 0.30 μmoles/min/ml) FPs were intermediate between control and ChCl groups. There were significant differences between normal and ChCl ($P < 0.001$), and Tris ($P < 0.05$). However, ovoperoxidase release was not significantly different between normal and KCl ($P > 0.35$).

Ovoperoxidase localization

Because ovoperoxidase is incorporated into the FE (Somers *et al.*, 1989) and more enzyme activity was observed in the FP of the low Na^+ treatments (see above), it is possible that the higher activity was not only due to

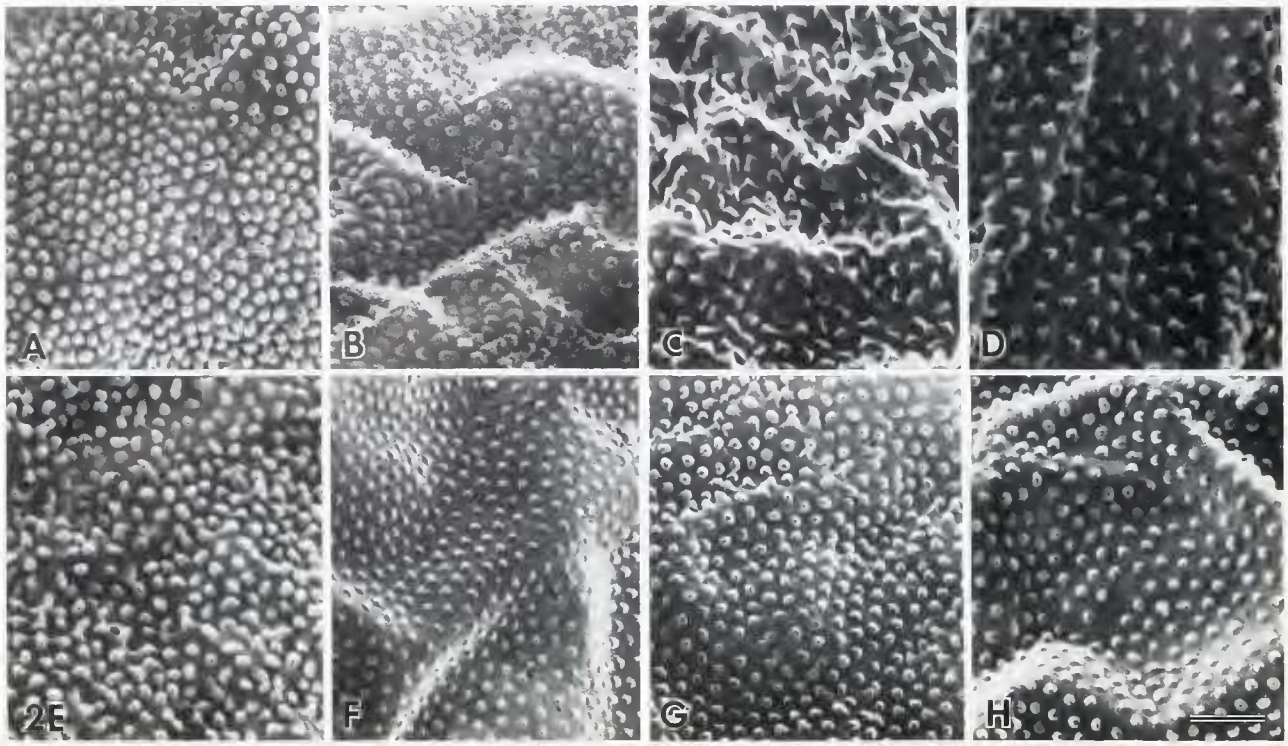

Figure 2. SEM of *Strongylocentrotus purpuratus* egg surfaces. A. VL of egg (unactivated) in normal Na^+-SW. B–D. FEs of normal Na^+-SW eggs at 1,3, and 60 min postactivation. E. VL of egg (unactivated) in ChCl-substituted-SW. F–H. FEs of ChCl-substituted-SW eggs at 1,3, and 30 min. The microvillar projections of the ChCl-substituted-SW eggs did not undergo the I–T transformation. Scale bar = 1 μm.

FE permeability, but that the ovoperoxidase was not incorporated efficiently into the structure of the FE. Therefore, DAB localization of ovoperoxidase was performed.

Comparing the TEM micrographs (Fig. 7) of FEs after DAB incubation, the normal SW FE (Fig. 7C) is conspicuously darker than that of the ChCl FE (Fig. 7D). Although both normal and ChCl FEs stained more intensively than the controls (Fig. 7A, B), the intensity of staining was higher in the normal FEs. Presumably, there was more ovoperoxidase incorporated into the FEs in normal Na^+-SW than in ChCl-substituted SW.

Discussion

Sea urchin eggs are excellent material for many biological studies because they can be harvested in large numbers, cultured in a well-defined medium (artificial seawater), and they develop synchronously. They are well suited for the study of extracellular self-assembly (Kay and Shapiro, 1985; Somers and Shapiro, 1989; Shapiro *et al.*, 1989). The complexity of the transition of the vitelline layer (VL) glycoprotein to the FE tempts one to try to dissect the myriad of sequential processes involved. The VL is not merely an inert cell coat, but it serves as a template or scaffolding upon which other proteins are assembled and intercalated under the influence of several enzymes. A requisite for proper structuralization is the presence of several ions in the seawater, *e.g.*, Ca^{2+}, Mg^{2+} (Carroll and Endress, 1982), Cl^- (Lynn *et al.*, 1988; Green *et al.*, 1990), and Na^+ (Schuel *et al.*, 1982). In the present study, we focused on the effects of Na^+-depletion on the elevation and structuralization of the FE.

There is some information on the sea urchin egg during fertilization in Na^+-depleted seawater. It was found that the fast block to polyspermy decayed concurrently with the retardation of the depolarization of the egg plasma membrane, a Na^+-dependent process (Jaffe, 1980; Schuel and Schuel, 1981). Additional investigations have shown that Na^+ accounts for the release of acid from the egg, resulting in an increased intracellular pH. This increase is necessary for increased protein synthesis, DNA synthesis, and cell division (Nishioka and Cross, 1978). In relation to the assembly of the FE, Schuel *et al.* (1982) demonstrated that low Na^+ (19 m*M*), ChCl-substituted seawater resulted in FEs that collapsed and failed to undergo normal structuralization, including the I to T transformation of the microvillar casts. The inhibition of the normal hardening process was attributed to the failure of CG structural proteins to impregnate or insert into the VL. However, this impairment of the hardening process is dis-

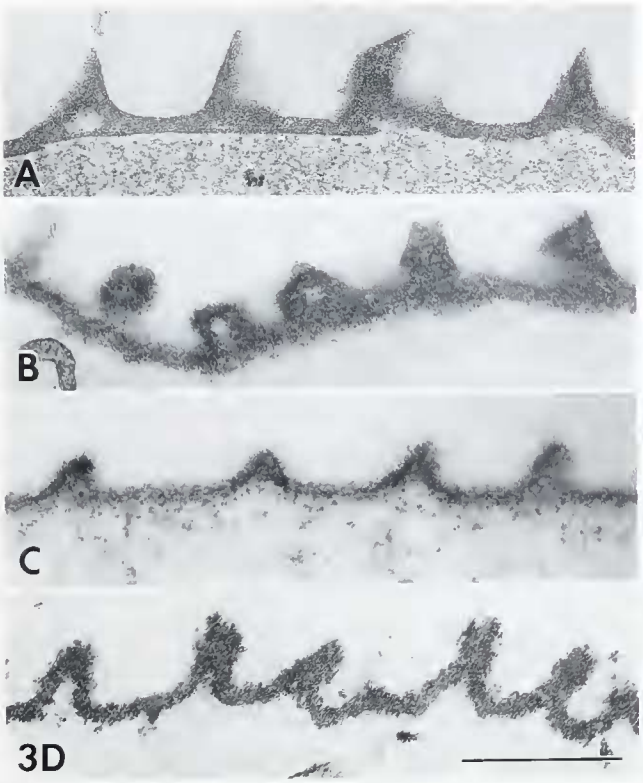

Figure 3. TEM of *Strongylocentrotus purpuratus* FEs. Eggs were fixed 15 min postactivation in the following SWs: A. normal Na^+. B. KCl. C. Tris. D. ChCl. Scale bar = 0.5 μm.

tinct from the phenomenon of "cross-linking," in that the latter is assayed by the disruption of FEs in urea. In their experiments, cross-linking was not affected. Furthermore, K^+ and Li^+ substituted for Na^+ in normal structuralization, while ChCl and Tris did not.

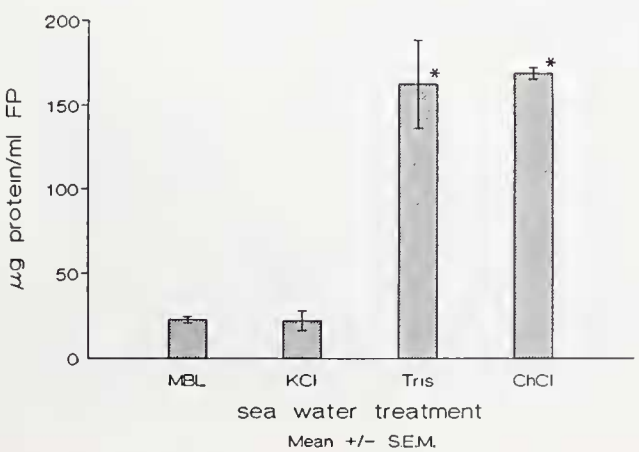

Figure 4. Protein release through FEs. Protein concentrations were determined by the Lowry assay. Each measurement represents the Mean ± S.E.M. of three trials. The * denotes a statistically significant difference from the control, normal SW.

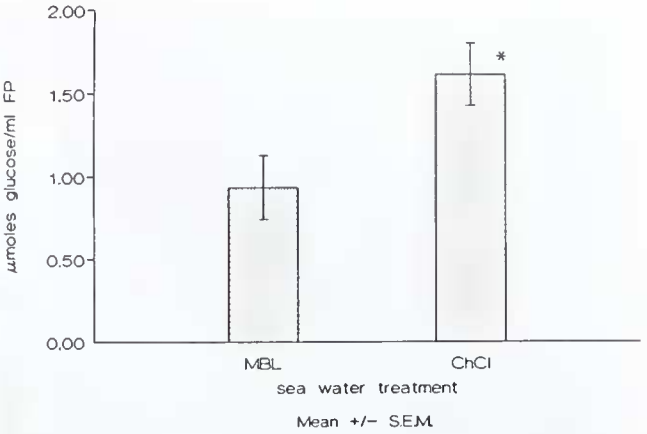

Figure 5. β-1,3-glucanase secretion. Glucanase activity released through FEs was assayed as described in Materials and Methods. The Mean ± S.E.M. are shown for four trials. The * denotes a statistically significant difference from the control, normal SW.

In the present study we lowered the Na^+ concentration to approximately 2 m*M* and observed an earlier collapse of the FE, 3 min as opposed to 30 min. It is not surprising that our FEs collapsed earlier than those of Schuel *et al.* (1982), because our Na^+ concentration was ten-fold less. Additional evidence of the failure of the sodium-deficient FEs to harden is that initially they expanded more than the normal FEs. This greater distension may also be related to the failure of proteins to insert into the FE, thereby raising the hydrostatic pressure in the perivitelline space (Schuel *et al.*, 1974; Green and Summers, 1980). However, within a few minutes, the FE began to shrink, suggesting that the FE was permeable to the secreted proteins, and this allowed for a decrease in the hydrostatic pressure

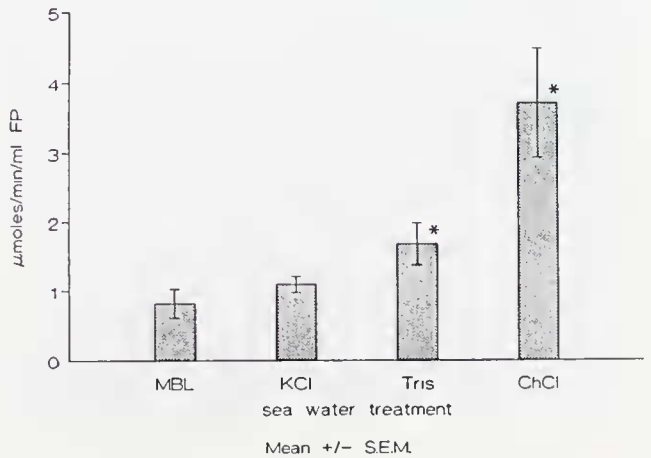

Figure 6. Ovoperoxidase secretion. Ovoperoxidase activity released through FEs was measured as described in Materials and Methods. Mean ± S.E.M. are shown (n = 3–7) and * denotes statistically significant differences from the control, normal SW.

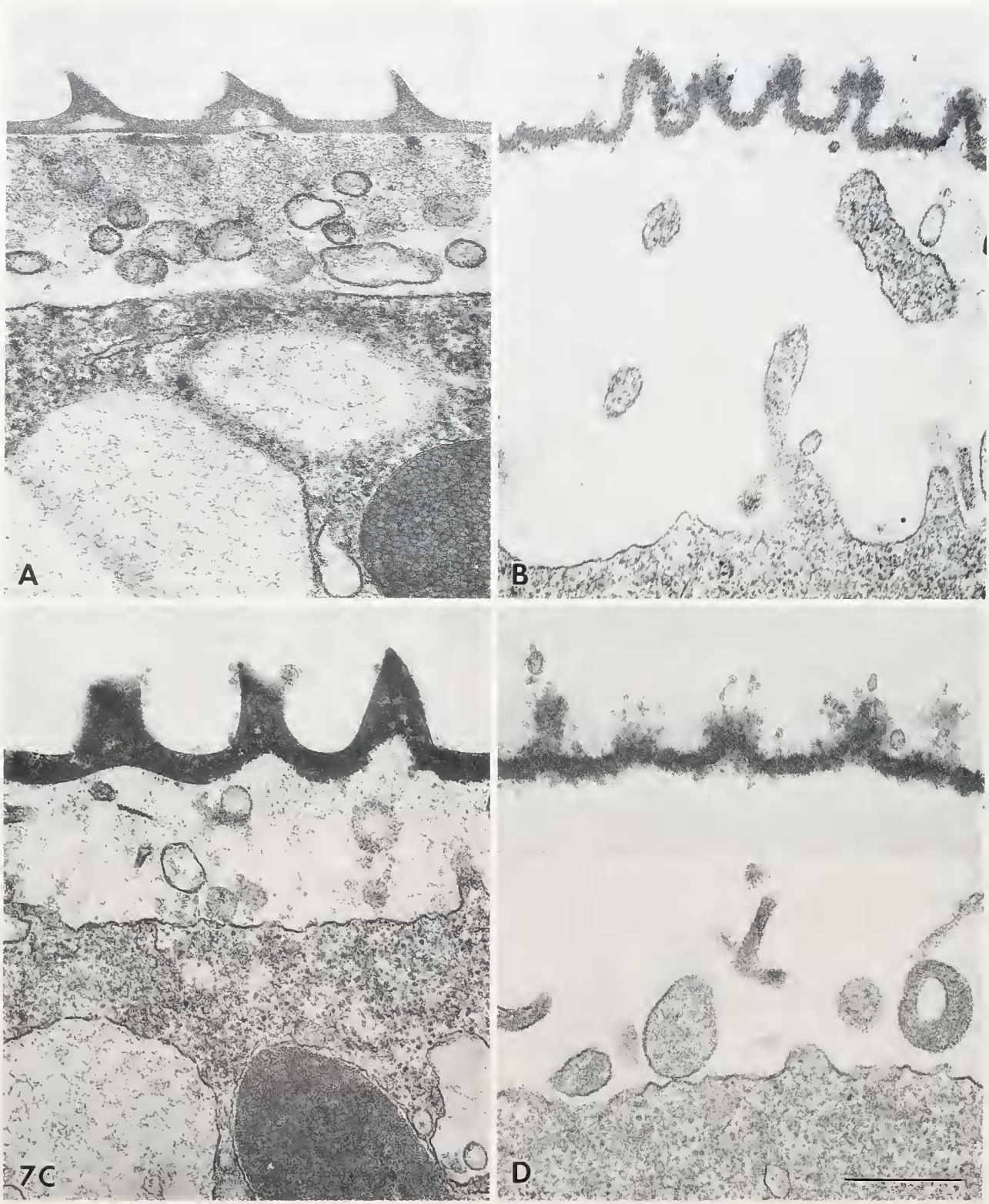

Figure 7. Ultrastructural localization of ovoperoxidase. The pointed T-form microvillar projections on normal Na^+-FE (15 min after activation) and the rounded I-form microvillar projections on ChCl-FE are demonstrated (A and B, respectively). After DAB localization of ovoperoxidase the FEs (C, normal Na^+; D, ChCl) stained more intensely than those of the controls (A and B) in both normal Na^+ and ChCl-substituted-SW. Furthermore, the staining of the normal Na^+ was darker than that of the ChCl. This suggests that less ovoperoxidase was incorporated into the FE of eggs activated in ChCl-substituted SW. D is a montage showing the FE and cortex of the same egg. Scale bar = 0.5 μm.

within the perivitelline space. This conclusion is strengthened by the fact that we observed a greater (approx. eightfold) increase in total proteins secreted into the ambient seawater in Tris and ChCl seawaters. Glucanase and ovoperoxidase secretions also increased significantly. Because ovoperoxidase is normally incorporated into the FE (Somers and Shapiro, 1989), the increase in soluble ovoperoxidase activity is probably related to both increased permeability and decreased incorporation of this enzyme into the nascent FE. The substitution of K^+ for Na^+ did not significantly interfere with hardening (structuralization), nor did it change protein or ovoperoxidase secretion significantly. This observation is consistent with the normal FE formation in K^+- or Li^+-substituted seawaters (Schuel *et al.*, 1982).

The results reported herein are similar to those obtained in Cl^--deficient seawaters (Lynn *et al.*, 1988; Green *et al.*, 1990). Moreover, our results are strikingly similar to those of Battaglia and Shapiro (1988), who reported similar findings when they inhibited egg surface transglutaminase activity with primary amines. This similarity suggests that both Cl^- and Na^+ may be important for the transglutaminase-catalyzed early cross-linking that occurs before the ovoperoxidase-catalyzed cross-linking. However, we can not ignore the possibility that the observed effects may be the result of ChCl or Tris **addition**, rather then exclusion of Na^+. This is a drawback to any substitution experiment. The size of the substituted ionic species may be important because K^+ and Li^+ are closer in size to Na^+ than are either Tris or ChCl. This question, too, remains to be resolved.

Another interesting observation of these experiments was the apparent paucity of hyalin in the perivitelline space of eggs activated in ChCl-substituted-SW (*e.g.*, Figs. 3A, D and 7A, B). This observation and the increased glucanase activity in the ambient sodium-deficient seawater may be related to the possibility that glucanase performs its major function on the hyaline layer of the egg (Wessel *et al.*, 1987) in a Na^+-dependent manner. This possibility awaits further experimentation.

Our results demonstrate that the ionic composition of the seawater significantly influences the formation of the FE in the sea urchin *S. purpuratus*. These results are consistent with other published reports. Moreover, the phenomenon of regulated extracellular assembly remains an intriguing field of study, to which the study of sea urchin FE formation can contribute.

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